

Stimulation of DNA synthesis in Jurkat cells by synergistic action between adenine and guanine nucleotides

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P₂-purinoceptor agonists stimulated the DNA synthesis of Jurkat cells via a pathway independent of cAMP and intracellular free calcium. The response was greatly enhanced by the synergistic action between adenine and guanine nucleotides, suggesting that binding sites of these nucleotides are different from each other, and the proliferation is stimulated by a novel interaction between adenine and guanine nucleotide receptors. The stimulatory effects of P₂-agonists on proliferation were completely abolished by cholera toxin and attenuated by pertussis toxin, which suggests that substrates for cholera toxin and pertussis toxin are involved in the proliferative pathways associated with P₂-purinoceptors.

P₂-purinoceptor; Proliferation; Jurkat cell

1. INTRODUCTION

It was reported that a variety of early responses including cAMP accumulation, an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$), and diacylglycerol release are associated with the activation of the signal transduction system in T lymphocytes [1–4]. On the other hand, experiments using cholera toxin and pertussis toxin have suggested that GTP binding proteins (G proteins) are involved in these signalings [4–8].

In the present study, we have found that the proliferation of human T lymphocyte Jurkat cells is stimulated by the synergistic action between adenine and guanine nucleotides. The results presently obtained show that P₂-purinergic agonists stimulate the proliferation independent of both cAMP accumulation and an increase in $[Ca^{2+}]_i$, and suggest the involvement of the toxin-sensitive G protein(s) in cell proliferation.

2. MATERIALS AND METHODS

2.1. Materials

Human T lymphocyte Jurkat cells were maintained in IMDM (Iscove's modified Dulbecco's Media, Gibco) supplemented with 10% fetal calf serum (FCS) as described [9]. cAMP assay kits were provided by Yamasa Shoyu. Pertussis toxin (IAP) and phosphodiesterase inhibitor Ro-20-1724 were provided by Kaken Pharmaceutical Co. and Nippon Roche, respectively. PGE₂, GTP γ S, ATP γ S, GDP β S, and ADP β S were obtained from Boehringer Mannheim. Fura 2/AM and cholera toxin were from Dojin and Seikagaku Co., respectively. 5'-N-ethylcarboxamideadenosine (NECA) and phenylisopropyladenosine (PIA) were from RBI. [Methyl-³H]thymidine (1.56 TBq/mmol, [³H]dThd) was from Amersham.

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2.2. Determination of cAMP accumulation

Cells were washed 3 times, followed by centrifugation at $150 \times g$ for 3 min, with ice-cold Tyrode HEPES buffer (137 mM NaCl, 3.3 mM KH₂PO₄, 0.7 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, 0.5% BSA, and 20 mM HEPES, pH 7.5), and resuspended in the same buffer. Cell suspensions (5×10^5 cells/ml) were then incubated with various agents in the presence of 0.3 mM RO-20-1724 at 37°C for 10 min. cAMP assay was carried out as described previously [10].

2.3. Fura/AM loading and measurement of $[Ca^{2+}]_i$

Jurkat cells (2×10^7 cells/ml) in IMDM supplemented with 1% BSA (loading buffer) were incubated with 3 μ M Fura 2/AM at 37°C for 15 min. The cell suspension was diluted 10 times with loading buffer and further incubated for 30 min. Cells were washed once with the loading buffer, resuspended in the same buffer, and maintained in a 30°C water bath until the measurement of $[Ca^{2+}]_i$. Prior to the spectrofluorometric experiment for the measurement of $[Ca^{2+}]_i$, cells were washed once and suspended in Tyrode HEPES buffer. Various agonists were added, and $[Ca^{2+}]_i$ was measured using a Hitachi F-2000 fluorescence spectrophotometer as described [11].

2.4. Measurement of DNA synthesis

Cells were cultured in 6-well plates of IMDM supplemented with FCS at a concentration of 2×10^6 cells/ml/well with various agents for 10–15 h prior to addition of [³H]dThd. The cultures were labeled by adding 2 μ M [³H]dThd (1 μ Ci/well) for 5–6 h. The cells were harvested onto Whatman glass fiber filters (GF/C) with Tyrode HEPES buffer and washed 3 times with 5% trichloroacetic acid. The radioactivity on the filters was measured in a liquid scintillation counter.

3. RESULTS AND DISCUSSION

3.1. Stimulation of [³H]dThd incorporation by purinergic agonists

Fig. 1 shows that ATP and GTP increased [³H]dThd incorporation in Jurkat cells in a concentration-dependent manner. These results suggest that there are purinoceptors related to cell proliferation on cell membranes. Effects of a variety of purinergic agonists on [³H]dThd incorporation were examined (Table I). The

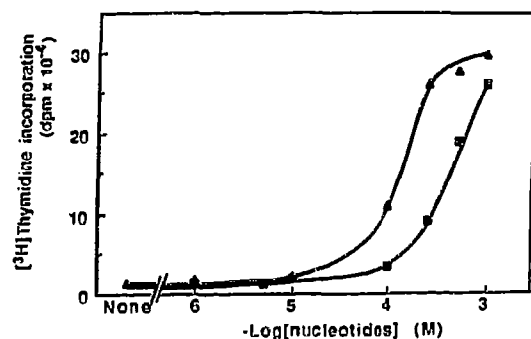


Fig. 1. Dose-response curves for ATP- and GTP-stimulated $[^3\text{H}]\text{dThd}$ incorporation. Cells were cultured with indicated concentrations of ATP (\blacktriangle) and GTP (\blacksquare) for 12 h. The cultures were further incubated with $[^3\text{H}]\text{dThd}$ for 6 h and used for measurement of $[^3\text{H}]\text{dThd}$ incorporation as described in section 2.

rank order of the response of $[^3\text{H}]\text{dThd}$ incorporation was $\text{ATP} > \text{GTP} = \text{ADP} > \text{GDP}$. ATP, which was the most effective at a concentration of 0.5 mM, increased the incorporation by approximately 20-fold, while non-hydrolyzable adenosine analogues NECA and PIA had no effect.

3.2. Relationship between cAMP accumulation, $[\text{Ca}^{2+}]_i$ and $[^3\text{H}]\text{dThd}$ incorporation

It has been reported that cAMP inhibits the lymphocyte proliferation [1,2], while intracellular Ca^{2+} is required for mitogenic stimulation [4,12,13]. In contrast, it was reported that cAMP acts as a mitogenic signal in some cell lines [14,15]. Hence, we examined whether or not stimulation of $[^3\text{H}]\text{dThd}$ incorporation by purinergic agonists is associated with cAMP levels, $[\text{Ca}^{2+}]_i$, or both. ATP and NECA increased cAMP levels dose-dependently (Fig. 2A), while both agonists also raised $[\text{Ca}^{2+}]_i$ (Fig. 2B). GTP was not effective on cAMP accumulation and an increase in $[\text{Ca}^{2+}]_i$ (Fig. 2A,B). These results suggest that stimulatory effects of ATP and GTP on $[^3\text{H}]\text{dThd}$ incorporation are unrelated to the increases in cAMP levels and $[\text{Ca}^{2+}]_i$.

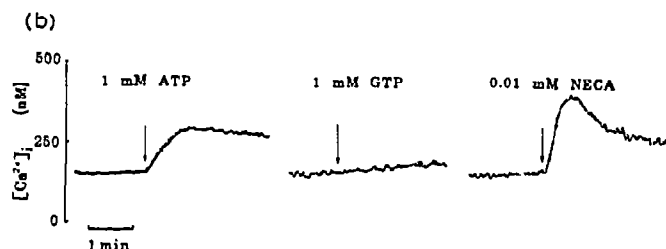
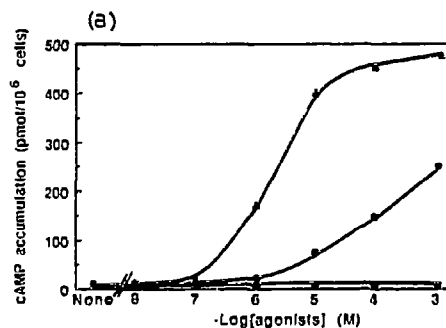


Fig. 2. Effects of ATP, GTP, and NECA on cAMP accumulation and $[\text{Ca}^{2+}]_i$. A. Cells were incubated with indicated concentrations of ATP (\bullet), GTP (\blacksquare), and NECA (\blacktriangle), and used for measurement of cAMP levels as described in section 2. B. Cells were loaded with Fura 2/AM, and then ATP, GTP, or NECA was added. Ca^{2+} -specific fluorescences were measured as described in section 2.

In order to confirm that cAMP and $[\text{Ca}^{2+}]_i$ are not associated with $[^3\text{H}]\text{dThd}$ incorporation, we measured changes in $[\text{Ca}^{2+}]_i$, cAMP levels, and $[^3\text{H}]\text{dThd}$ incorporation in response to a variety of nucleotides including non-hydrolyzable analogues, PIA, and cAMP elevators (Fig. 3, Table II). Adenine nucleotides, PGE_2 and forskolin increased cAMP levels, accompanying with an increase in $[\text{Ca}^{2+}]_i$, but PGE_2 and forskolin did not increase $[^3\text{H}]\text{dThd}$ incorporation similar to NECA and PIA. In contrast, guanine nucleotides, UTP, and ITP increased neither cAMP levels nor $[\text{Ca}^{2+}]_i$. These agonists, however, increased $[^3\text{H}]\text{dThd}$ incorporation, although the effects of UTP and ITP were much weaker than those of guanine nucleotides. These results indicate that P_2 -purinergic agonists increase $[^3\text{H}]\text{dThd}$ incorporation independent of cAMP accumulation and $[\text{Ca}^{2+}]_i$, while P_1 -purinergic agonists and cAMP elevators did not increase it. The observations that cAMP accumulation is accompanied by an increase in $[\text{Ca}^{2+}]_i$ are consistent with the report that agents elevating cAMP raise $[\text{Ca}^{2+}]_i$ in Jurkat cells [11].

3.3. Synergistic effects of adenine and guanine nucleotides on $[^3\text{H}]\text{dThd}$ incorporation

As described above, ATP increased cAMP levels and $[\text{Ca}^{2+}]_i$, while guanine nucleotides, UTP and ITP did not increase them, suggesting that adenine nucleotide binding sites are different from other nucleotide bind-

Table I

The effects of purinergic agonists on $[^3\text{H}]\text{dThd}$ incorporation	
Agonists	$[^3\text{H}]\text{dThd}$ incorporation (cpm/well)
None	17435 \pm 956
0.5 mM ATP	337531 \pm 10377
0.5 mM ADP	213197 \pm 9211
0.5 mM GTP	241029 \pm 10822
0.5 mM GDP	48366 \pm 1855
10 μM NECA	19374 \pm 1228
10 μM PIA	18311 \pm 701

Cells were cultured with various agonists for 12 h. The cultures were further incubated with $[^3\text{H}]\text{dThd}$ for 6 h and used for measurement of $[^3\text{H}]\text{dThd}$ incorporation as described in section 2. Data represent the mean \pm SE from quadruplicate determinations.

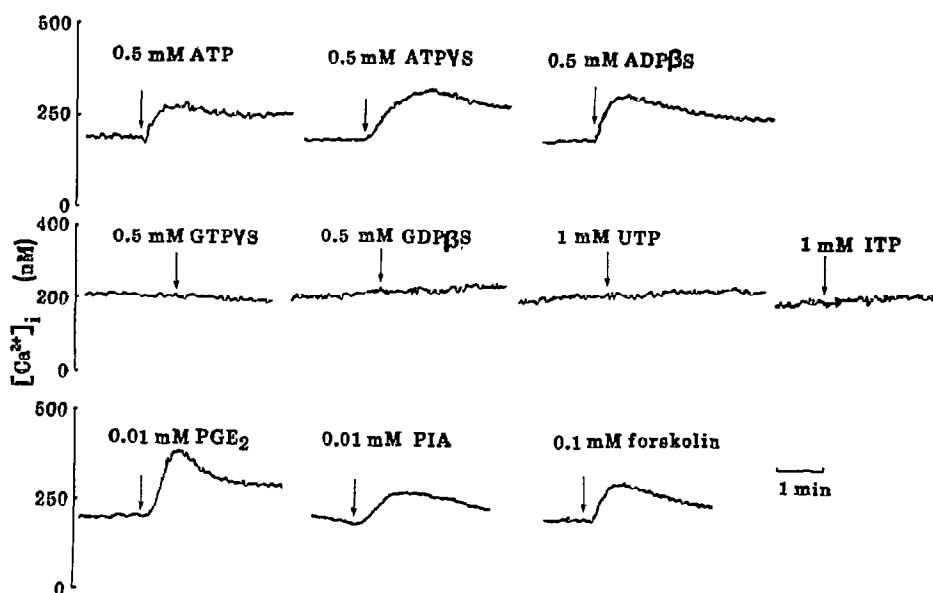


Fig. 3. Effects of nucleotides, adenosine analogues, and cAMP elevators on $[Ca^{2+}]_i$. Cells were loaded with Fura 2/AM, and then various agents were added. Ca^{2+} -specific fluorescences were measured as described in the legend to Fig. 2B.

ing sites. Then we examined the combination effects of adenine and guanine nucleotides on $[^3H]dThd$ incorporation. $GDP\beta S$ slightly increased $[^3H]dThd$ incorporation by 2.0-fold at a low concentration of $100 \mu M$. The dose-dependent stimulatory effects of ATP and $ADP\beta S$ on $[^3H]dThd$ incorporation were markedly enhanced by the addition of $100 \mu M$ $GDP\beta S$. These actions enhanced by $GDP\beta S$ were attenuated at higher concentrations of ATP (Fig. 4). ATP slightly increased by 1.9-fold at a low concentration of $50 \mu M$. The stimulatory effects of $GTP\gamma S$ and $GDP\beta S$ were markedly enhanced by the addition of $50 \mu M$ ATP, and the enhancement by ATP was also attenuated at higher

concentrations of $GDP\beta S$ (Fig. 5). These results indicate that receptors of adenine and guanine nucleotides are different from each other, and stimulation of both receptors brings about a synergistic effect through a novel interaction on $[^3H]dThd$ incorporation. Adenine and guanine nucleotide-stimulated $[^3H]dThd$ incorporation was unaffected by the addition of all concentrations of NECA tested (data not shown).

It has been reported that ATP causes synergistic enhancement of proliferation when combined with growth factors such as PDGF, $TGF\alpha$, and EGF in mouse fibroblasts [16,17]. We found that low concentrations of adenine and guanine nucleotides synergistically affect the proliferation of Jurkat cells. It seems to be implied that these nucleotides are effective on proliferation at physiological conditions.

Table II

Relationship between cAMP accumulation, $[Ca^{2+}]_i$ and $[^3H]dThd$ incorporation

Agents	cAMP accumulation (pmol/ 10^6 cells)	$[^3H]dThd$ Incorporation (cpm/ 10^6 cells)
None	6.4 ± 1.2	15089 ± 606
$0.1 \text{ mM } ADP\beta S$	26.7 ± 1.0	40262 ± 1299
$0.1 \text{ mM } GDP\beta S$	5.9 ± 0.9	33426 ± 1027
$0.1 \text{ mM } ATP\gamma S$	130.3 ± 7.3	114555 ± 4474
$0.1 \text{ mM } GTP\gamma S$	6.8 ± 0.6	50221 ± 2166
$1.0 \text{ mM } UTP$	7.7 ± 1.4	38488 ± 2527
$1.0 \text{ mM } ITP$	7.5 ± 1.0	37765 ± 1678
$10 \mu M$ Forskolin	1033.7 ± 58.7	14454 ± 823
$10 \mu M$ PIA	51.3 ± 2.1	14311 ± 922
$10 \mu M$ PGE_2	181.5 ± 15.5	17155 ± 717

cAMP accumulation: Cells were incubated with various agents and used for measurement of cAMP levels as described in section 2. $[^3H]dThd$ incorporation: Cells were cultured with indicated concentrations of agent for 12 h. The cultures were further incubated with $[^3H]dThd$ for 6 h and used for measurement of $[^3H]dThd$ incorporation as described in Table I. Data represent the mean \pm SE from quadruplicate determinations.

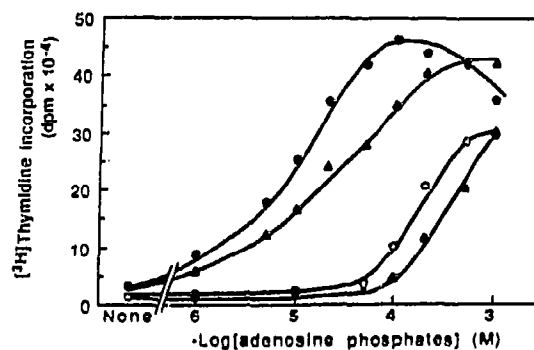


Fig. 4. Synergistic effect of $GDP\beta S$ on ADP- and ATP-stimulated $[^3H]dThd$ incorporation. Cells were cultured with the indicated concentrations of ADP (Δ , \blacktriangle) and ATP (\circ , \bullet) with (closed symbols) or without (open symbols) $100 \mu M$ $GDP\beta S$ for 13 h. The cultures were further incubated with $[^3H]dThd$ for 6 h and used for measurement of $[^3H]dThd$ incorporation as described in the legend to Fig. 1.

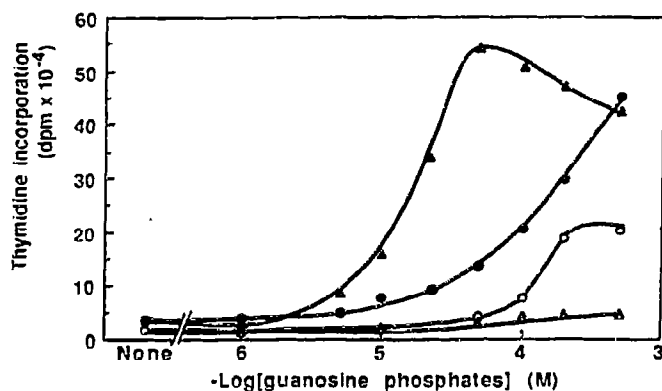


Fig. 5. Synergistic effect of ATP on GDP- and GTP-stimulated [^3H]dThd incorporation. Cells were cultured with the indicated concentrations of GDP γ S (Δ , \blacktriangle) and GTP γ S (\circ , \bullet) with (closed symbols) or without (open symbols) 50 μM ATP for 13 h. The cultures were further incubated with [^3H]dThd for 5 h and used for the measurement of [^3H]dThd incorporation as described in the legend to Fig. 1.

3.4. Effects of cholera toxin and pertussis toxin on $[\text{Ca}^{2+}]_i$ and [^3H]dThd incorporation

The effects of cholera toxin- and pertussis toxin-treatment on [^3H]dThd incorporation were examined. The stimulatory effect of the combination of ATP and GDP γ S on [^3H]dThd incorporation was abolished in cholera toxin-treated cells (Fig. 6A). An increase in $[\text{Ca}^{2+}]_i$ induced by ATP was also abolished in cholera toxin-treated cells (Fig. 6B). One may consider that the marked increase in cAMP levels and inhibition of calcium release from intracellular calcium stores by cholera toxin-treatment lead to suppression of [^3H]dThd incorporation similar to that of other types of cells [18,19]. NECA and PGE $_2$, which greatly increased cAMP levels, did not, however, suppress [^3H]dThd incorporation induced by ATP (data not shown), suggesting that the inhibitory effect of cholera toxin on proliferation is independent of cAMP accumulation.

It has been demonstrated that cholera toxin cAMP-independently causes ADP-ribosylation of a 25-kDa protein in rat liver [20] and inhibition of chemotaxis in the mouse macrophage RAW 264 cell line [21]. In addition, it was proposed that cAMP-independent G protein, distinct from G $_{\text{sa}}$ is involved in T lymphocyte activation [5,8]. Hence, the cholera toxin-sensitive and cAMP-independent G protein may be involved in the proliferation pathway induced by P $_2$ -purinergic agonists. Furthermore, IAP suppressed [^3H]dThd incorporation enhanced by ATP plus GDP γ S by approximately 40% (Fig. 6A). In contrast, the increases in $[\text{Ca}^{2+}]_i$ by ATP and PIA were enhanced in IAP-treated cells (Fig. 6B,C). These results support our finding that [^3H]dThd incorporation by P $_2$ -purinergic agonists is unrelated to an increase in $[\text{Ca}^{2+}]_i$. In addition, the above results are consistent with a report by Rosoff et al. [6] that IAP causes a rapid rise in $[\text{Ca}^{2+}]_i$. They also

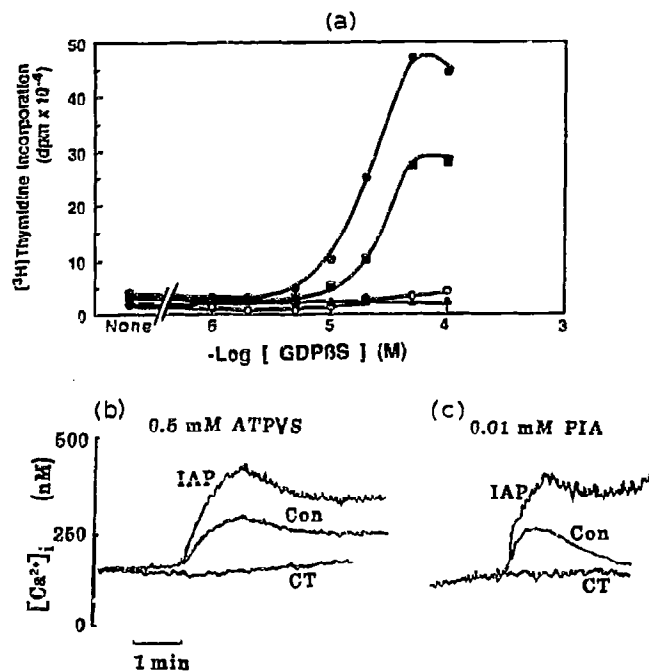


Fig. 6. Effects of cholera toxin- and IAP-treatment on [^3H]dThd incorporation and $[\text{Ca}^{2+}]_i$. Cells were cultured without (\circ , Con) or with cholera toxin (100 ng/ml) (\blacktriangle , CT) or IAP (100 ng/ml) (\bullet) for 10 h. A. Each cell was cultured in the presence of 50 μM ATP plus indicated concentrations of GDP γ S for 12 h. The cultures were further incubated with [^3H]dThd for 5 h and used for measurement of [^3H]dThd incorporation as described in the legend to Fig. 1. B, C. Each cell was loaded with Fura 2/AM, and then ATP γ S (B) or PIA (C) was added. Ca^{2+} -specific fluorescence were measured as described in the legend to Fig. 2B.

showed that IAP itself stimulates proliferation via an increase in $[\text{Ca}^{2+}]_i$ and production of diacylglycerol in Jurkat cells [6,22]. However, under our conditions where cells were treated with IAP for 10–15 h, IAP did not stimulate the proliferation.

The physiological role(s) of cAMP accumulation and an increase in $[\text{Ca}^{2+}]_i$ induced by purinergic agonists are unclear at present. In this connection, it is interesting to note that cAMP increased by PGE $_2$ modulates the immune response in T lymphocytes [11]. Further study will be needed to clarify whether or not P $_2$ -purinergic agonists modulate the immune response similar to PGE $_2$.

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